Validation of Multiplex Real-Time PCR Tests for Intestinal Parasites

Presented by Louis Bian
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Under the supervision of Chun Wang, M.S.
Molecular Biology Acting Team Lead at the Texas Department of State Health Services

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Dr. Leanne H. Field
Supervising Professor
Honor's Advisor in Public Health

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ABSTRACT

Introduction

Parasitic infections are one of the most common human infections around the world, especially in refugees. The current standard of diagnosis for parasitic infections is microscopy of stool specimens. However microscopy has several limitations, some of which include time, inexperience and subjectivity in diagnosis. To improve the specificity, sensitivity and turn-around time of the diagnosis method, multiplex real-time PCR (RT-PCR) diagnosis has recently been applied in this field. Multiplex RT-PCR can simultaneously amplify and detect the presence of multiple parasites in one sample in one run.

Methods

The study population was comprised of 32 refugees arriving in Texas between the years of 2010 and 2012 that were infected with *Giardia lamblia, Dientamoeba fragilis,* and/or *Strongyloides stercoralis*. The probe/primer mixes to identify these parasites using multiplex RT-PCR were first validated in uniplex assays and then tested for effectiveness in multiplex assays.

Results

The results for the uniplex assay suggest that the *G. lamblia* (100% agreement between uniplex and microscopy) and *D. fragilis* (82.4% agreement) probe/primer mixes were validated, but the *S. stercoralis* (0% agreement)

probe/primer mix was not. A specific type of multiplex, known as a duplex, was conducted to identify the parasites *G. lamblia* and *D. fragilis*. In the multiplex assay, *G. lamblia* had a 100% agreement while *D. fragilis* had an 85.7% agreement. In the case of co-infections, there was a 100% agreement to identify the presence *G. lamblia* and a 66.7% agreement to identify *D. fragilis*. There was a 0% false positive rate for both uniplex and multiplex assays for all parasites.

Conclusion

It was concluded that the multiplex RT-PCR assay to diagnose these two parasites was successful and the results agreed with microscopic diagnoses.

INTRODUCTION

Public Health Significance of Parasitic Diseases

Parasites infect approximately one billion people, making them one of the most common human infections (15, 25). The burden of parasites is greatest in the tropic and sub-tropic areas of the world, though they are not limited to these geographic locations. Neglected Tropical Diseases (NTDs) are diseases that are endemic among low-income and impoverished populations. Ten of the top 13 of NTDs are caused by parasites (24, 26). The top three of these have a cumulative prevalence of two billion and an at-risk population of 10.6 billion (24). Despite the fact that the parasitic NTDs affect the poor in disproportionally high numbers and account for a burden that is at least equivalent to tuberculosis and malaria, less than 1% of overseas development aid is used to control these diseases (18, 24, 35).

Domestically, about 65 million people in the United States (US) are infected with intestinal parasites, and a vast majority of them are refugees (cdc.gov). A refugee is defined as a person who is unable or unwilling to return to his/her home country due to fear of persecution based on race, gender, belief, nationality, and/or political view (cdc.gov). All refugees are required to undergo a medical examination before departure from their home country and upon arrival in the US (44). Refugees can be denied entry based on health reasons, such as carrying a disease of significant public health importance or a mental condition associated with harmful behavior (44).

Prevalence rates for parasitic infections in refugees entering the US range from a low of 8% to a high of 86% (cdc.gov). There are multiple reasons for the wide

range of prevalence including socioeconomic status, geography, dietary habits, living conditions, and standards of the country from which the refugees are arriving (51). In addition, different methods of detection have been shown to result in different detection rates. An example was the detection of parasites in Cambodian refugee populations in New York, where the prevalence ranged from 31% to 86% due to methodological differences among parasitic diagnostic tests (cdc.gov).

As introduced previously, before refugees leave their home country for the US, they are required to undergo a pre-departure examination that includes a physical, diagnostic test (tuberculosis, syphilis, etc.), and vaccination check by an overseas panel physician, an appointed doctor by the US (cdc.gov) (5, 45, 53). To reduce the parasitic prevalence rate in refugees, the CDC recommends a 600 mg presumptive Albendazole treatment before departure to the US, and the World Health Organization includes four anthelminthic drugs on their essential medicine list (30, 48).

After refugees arrive in the US, a domestic refugee medical examination is conducted (8). In studies by Chang (2013) and Shah (2008), refugees in 2010, when compared to refugees in 2008, were less likely to be infested with helminths partly due to the CDC recommended presumptive Albendazole treatment (12, 43). However, the effectiveness of this treatment varies from high to minimal depending on the parasitic species (48).

Since 1975, more than 3 million refugees have entered the US (33). Refugees can introduce new or existing diseases to areas in the US where prevalence rates are low (6, 14, 41). Refugees are typically referred to Preferred Communities

throughout the US that have opportunities for employment, independence and have support for special needs populations (cdc.gov). Upon arrival in such communities, refugees undergo different examinations dependent upon the state to which they are relocated. Typically, local health departments and private physicians carry out these examinations.

From 2000 to 2011, over 600,000 refugees arrived in the US from more than 60 different countries. Most refugees arrived from Cuba or the former Soviet Union and were relocated to either Florida, California or Texas (14).

Texas Refugee Demographic

According to the Texas Department of State Health Services (DSHS) 2010 Refugee Health Report, over 9,000 official refugees enter Texas annually. In 2010, the majority of refugees arrived from three countries: Burma (28%), Iraq (22%), and Bhutan (12%). Houston, Dallas, and Fort Worth are the major settlement areas for refugees and these three cities receive about 70% of all refugees that come into Texas. Thirty-seven percent of the arriving refugees in 2010 were between 18-32 years of age and the male to female ratio was 1.4 to 1 (dshs.state.tx.us).

Parasites

Ninety percent of refugees arriving in Texas were screened for intestinal parasites in 2010, and of those, 41% were found to be positive for at least one parasitic infection; 18% were prescribed presumptive treatment. Only 6% of all arriving refugees had received documented overseas treatment for parasites. Of

those with parasitic infections, *Giardia lamblia*, *Entamoeba histolytica,* and *Dientamoeba fragilis* accounted for 42%, 25% and 17% of all infections, respectively (dshs.state.tx.us).

Profile of Parasites

Geographically, parasitic infections typically occur along the equatorial/tropical latitudes and dense urban populations (24). Records of parasitic diseases date back to 'biblical times', and as such they are not considered as emerging diseases (24). Parasitic infections tend to be chronic, disabling diseases that cause great disease burden (morbidity), but low death rates (mortality). In addition, many societies stigmatize people with parasitic infections and indigent populations are kept impoverished due to the combined morbidity and stigma associated with parasitic infections (24).

The focus of this research project was three intestinal parasites *G. lamblia*, *D. fragilis*, and *S. stercoralis*. Recent research papers emphasize the importance of these intestinal parasites. A study in California concluded that *G. lamblia* and *D. fragilis* were the two most common protozoan infections in refugees (21). Another study found that in the absence of adequate treatment, *S. stercoralis* can persist for prolonged periods of time (19). A brief physical description of each of these parasite including its life cycle, disease description, and treatment for each disease is included below.

G. lamblia is an flagellated anaerobic protozoan parasite that colonizes the upper part of the small intestines and may even penetrate into the gallbladder of several vertebrates (29). The trophozoite form ranges from 9 to 21μm in length and 5 to 15 μm in width, while the cyst form ranges from 8 to 14 μm in length by 7 to 10 μm in width. Both forms do not consistently appear in stool specimens of patients; there are high patterns (present in almost all stools), low patterns (present in only about 40% of stool specimens) and mixed patterns (alternations between the previous two within a 1 to 3 week period). It can take anywhere from 10 to 36 days for *G. lamblia* parasites to be detected in stool specimens using conventional diagnostic techniques (29, 32).

A graphical depiction of *G. lamblia*'s lifecycle is shown in Figure 1. This parasite alternates between a trophozoite (active, feeding stage) and a cyst (dormant stage). *G. lamblia* is infective only in its cyst form. Infection with *G. lamblia* usually begins with ingestion of the cysts found in feces contaminated food or water, or through contact with disease vectors such as flies. The cysts are able to survive for several months in cold water. Once cysts reach the small intestines, they release two trophozoites each that attach to the small intestine lining and multiply through binary fission. Upon nearing the colon, encystation occurs and cysts are the most common form found in non-diarrheal feces. Diarrheal feces contain fewer cysts. Once released, the cysts are immediately infectious. It is unknown how animal reservoirs may affect transmission of *G. lamblia* in humans (29, 32).

Symptoms of the disease giardiasis include diarrhea, malaise, excessive gas, nausea, epigastric pain and upset stomach. However, infections are often completely asymptomatic. Children are more vulnerable to infection and experience more severe symptoms. Giardiasis occurs worldwide and is the most common parasitic infection in the US. It is considered to be the major cause of diarrheal outbreaks, usually due to contaminated water supplies. Children under 5 years of age and childbearing women, both of which are exposed at day care centers, are the most vulnerable to transmission of giardiasis. However, giardiasis is usually not a life-threatening disease even in those who are immunocompromised (29, 32).

First line treatments of giardiasis include metronidazole and tinidazole. Nirazoxanide is a liquid formula suitable for children. Additional therapies include albendazole and mebendazole. *G. lamblia* cysts are resistant to filtration and chlorination, but double strength iodine solutions for 20 minutes have been shown to be effective at killing them (29).

D. fragilis is a single-celled parasite found in the large intestine of humans, dogs, and gorillas. It can range from 3 to 18 μm in diameter and there is no known cyst form of the parasite. *D. fragilis* has rarely been seen to ingest red blood cells and almost never invades tissue. A unique characteristic of *D. fragilis* is that it does not survive very long in stool outside the host body. Prevalence rates for *D. fragilis* range from 1.5% to 20%, but it is very probable that the true rate in most areas is not known, because significantly higher prevalence rates are found when stool specimens are immediately preserved in polyvinyl alcohol (29, 32).

Figure 2 presents a diagrammatic representation of the life cycle of *D. fragilis*. The complete life cycle of this parasite is not completely understood; it is assumed to be transmitted by way of intestinal nematodes (29). The lack of an infective cyst stage common to most other parasites further shrouds the mode of transmission. Based on clinical data, transmission is believed to occur through the fecal-oral route and co-transmission with helminth eggs is another possibility (29, 32).

Symptoms of dientamoebiasis include abdominal pain, diarrhea, weight loss and fever (29). There is a higher coincidence rate of dientamoebiasis with enterobiasis that cannot be explained by chance alone. Low infection rates among homosexual men in the Oakland suggest that the disease is not transmitted via ordinary fecal-oral route (29, 32).

Iodoquinol is the main drug used to treat dientamoebiasis, and tetracycline is an alternative. In cases where these two drugs do not work, paromomycin is a second line drug that has been found to be very effective (29).

S. stercoralis is a parasitic nematode that is generally ranges from 1 to 2 mm long and only infects humans. *S. stercoralis* can often infect the lung as well as the large intestine depending on the migratory route. Different *Strongyloides* species can also infect cats and dogs, but these species are generally host specific although human infections from a dog have been documented (29). Strongyloidiasis differs from hookworm infections in that while most hookworm infections die out over time, hosts can be infected by *S. stercoralis* for an indefinite amount of time (29, 32).

A graphic representation of *S. stercoralis*'s life cycle is shown in Figure 3. The life cycle is complicated; *S. stercoralis* alternates between a free-living form and a parasitic form. The free-living form can penetrate the skin of any potential host with which it comes into contact. Once inside the host, female larvae develop and burrow into the intestinal lining where they lay eggs. This dual life cycle allows *S. stercoralis* to live in unfavorable conditions outside a host for many years until contact with a suitable host (29, 32).

Symptoms of strongyloidiasis include lesions around the penetration point of the larvae, burning pain, sepsis, weight loss and ulcers. In immunocompromised or severely malnourished patients, strongyloidiasis can enter a hyperinfection state, which includes fever, dyspnea, respiratory failure and hemoptysis (39). The mortality rate for the hyperinfection state can reach up to 90% (34). A unique phenomenon of strongyloidiasis is auto-infection. In auto-infection, the larvae develop in the gut, circulate back up to the lungs and then migrate back down into the small intestine, starting the whole cycle over again. The numbers of migratory larvae can grow so large that they will cause injury to other vital organs, including the liver, heart, pancreas, kidneys and/or central nervous system. Even in areas where strongyloidiasis is not endemic, it can persist for years in a host (again, due to auto-infection) (37). Examples of such infections included prisoners of war that were detained in WWII, who contracted the parasites and were still infected 65 years later (29, 32).

Treatment options for strongyloidiasis include albendazole and ivermectin. Cases of hyperinfection require higher doses of these drugs. Ivermectin has also been found to be very successful in eliminating strongyloidiasis (29).

Diagnostic Techniques for the Identification of Intestinal Parasites

The standard method of laboratory diagnosis for intestinal parasites is light microscopy. First, a stool specimen is collected from the patient preferably within an hour after defecation. It is recommended three specimens be collected spaced at least 48 hours apart (cdc.gov). Stool specimen consistency before preservation is critical to the parasite identification process. Intestinal protozoan parasite trophozoites (easier to identify than cysts) are found in liquid or soft stool, and almost never found in fully formed stool. Protozoan cysts, on the other hand, are generally found in fully formed stool, and rarely found in liquid or soft stool (29). Adding a 10% formalin and polyvinyl alcohol preservative solution in a 1:3 stool to preservative ratio preserves these specimens. This solution allows for a long shelf life at room temperature. Helminth and other macroscopic parasites can be found on the surface of unpreserved stool specimen (29).

Three common techniques are used to identify parasites in stool specimens. The easiest way to detect trophic forms of parasites is a direct wet mount (C. Snider, personal communication). This involves mixing a small portion of the feces (preferably feces that have just been collected and not preserved) with saline on a clean microscopic slide. Then, the slide is checked under a light microscope for

parasites, and if needed, it is stained to enhance contrast (especially for cysts) (47). However, staining will kill most trophozoites, thus, they might be unidentifiable after staining.

A second technique used to detect intestinal parasites is sedimentation/flotation separation. This technique takes advantage of the differing densities between eggs/cysts and the suspending liquid. Thus, when spun at high speeds, the eggs/cysts will separate from the suspending liquid (2). In sedimentation, the eggs/cysts will concentrate in a pellet at the bottom of a tube, while in flotation, the eggs/cysts will rise to the surface of the suspending liquid. The concentrated specimen can then be collected and examined for the presence of parasites, eggs and/or cysts. The addition of a stain may also be used to assist with the detection of parasites (13, 29). Additionally, a combination of these three techniques may be used to ensure accuracy.

A third technique, permanent stained slides, is the most accurate of these three detection techniques. This technique involves smearing a thin film of feces (preferably fresh) onto a clean slide and then permanently staining it in order to reveal much more detail than a direct wet mount (17). This method alone has been proven to detect a significantly higher percentage of parasites when compared to just direct examination or concentration techniques (29). The use of a permanent stain is recommended as a part of any standard stool examination for parasites.

Advantages and Disadvantages of Microscopy

Microscopic techniques for the identification of parasites are limited in several ways. In a study on *Plasmodium* spp., Barber and coworkers (2013) documented that it was difficult to differentiate closely related species via microscopy due to similar morphologies (3). The identification of parasites microscopically is selective and depends on the skill of the laboratory professional in evaluating the direct wet mount, concentrated solution, or permanently stained slides. There are many reasons for this variability, including the limited period that parasites are shed, the differing levels of experience of the microscopists, improper handling of the specimen, etc.. In addition, it is much more difficult to diagnose intestinal parasites in fecal specimens when only one specimen is evaluated (29).

The sensitivity of a diagnostic test is the proportion of tested positives in relation to true positives. Reported sensitivity for the detection of parasites by microscopy varies widely in literature, ranging from <10% to 85% according to different sources (11, 46). In addition, detection rates are highly variable depending on the parasitic species and there are difficulties distinguishing between closely related parasites. Microscopic specificity, the proportion of tested negatives in relation to true negatives, suffers a similar fate with reported ranges of 10% to 50%, varying widely based on different sources (11, 46).

An alternative to microscopy for parasitic diagnosis is serologic testing. This involves testing a serum specimen for the presence of specific antibodies produced

by the body due to the presence of a parasitic infection. Serologic testing is usually used in conjunction with a traditional stool examination (29). Serologic tests require that the patient's immune system be functional and reacting to an active infection, meaning it is less effective for patients that were very recently infected or are immunocompromised (29). Recent studies have revealed that serological testing is a useful tool in monitoring treatment of infections and has a higher detection rate for *S. stercoralis* than microscopic stool exams (7, 9, 12).

The detection of intestinal parasites can be considerably improved with respect to specificity and sensitivity by the use of molecular-based tests such as the polymerase chain reaction (PCR) (29). PCR is based on nucleic acid hybridization and the reaction, assisted by primers, amplifies the nucleic acid sequences. Primers target very specific sequences and different sequences can be amplified using different primers. Once bound, these primers assist with initiation of elongation, which produces a complementary DNA strand. As these strands separate, the primers re-attach to the complementary DNA strands, and the process repeats itself (1).

To detect the presence of a specific DNA strand, complementary probes are used. These probes bind to complementary sequences, and they contain a fluorescent protein that will fluoresce under specific conditions. The detection of fluorescence indicates the presence of the probe, and that specific nucleic acid sequences are present in the sample (1). The addition of fluorescent probes to target a specific sequence while simultaneously amplifying the sequence is known

as real-time PCR, as opposed to traditional PCR where the nucleic acid sequence is just amplified by primers (16, 28, 36). Investigations have shown that real-time PCR can detect microorganisms with high specificity and sensitivity (up to 100%) (49). Using probes that fluoresce at different wavelengths in a single assay, a multiplex real-time PCR, makes it possible to identify multiple species or multiple pathogens simultaneously. The detection of different fluorescent wavelengths enables the identification of which specific species or pathogens are present in the specimen.

The Essential Steps in RT-PCR

The first step in RT-PCR is extraction of the DNA from the stool specimens. A lysis buffer is used to expose the parasite's DNA in the stool specimen. Then, proteins, RNA and other miscellaneous non-DNA molecules are removed from the sample, usually through degradation with an enzyme. Inhibition of DNases is also done to prevent the degradation of the purified DNA.

The second step in RT-PCR is PCR amplification and detection of parasites. If a parasite is present in a stool specimen, then the corresponding primer will assist with the replication of a nucleic acid sequence unique to that parasite, and then a corresponding probe will attach to the sequence. If the machine detects the specific fluorescence wavelength of that probe, then the sample will be positive for the parasite corresponding to that probe. Testing for one parasite at a time in a single run in this fashion is a technique known as uniplex PCR. Testing for multiple

parasites in a single run with matching primers and probes (of different wavelengths) is a technique known as multiplex PCR (42).

Advantages and Disadvantages of RT-PCR

The combination of real-time PCR and multiplexing offers many advantages, including an increase in sensitivity, specificity and turn-around-time (4). Theoretically, the DNA from even a single parasite can be detected due the amplification of DNA. Real-time multiplex PCR eliminates many sources of human error such as inexperience, overburden, morphologically ruined samples, etc. (38). Disadvantages to this method include costs, an overlap in emission spectra (if the fluorescence wavelengths are too close), and the vulnerability of samples due to improper handling (31).

Several research studies have demonstrated that a multiplex real-time PCR assay can be used with confidence to simultaneously identify *E. histolytica*, *Cryptosporidium parvum* and *G. lamblia*. When compared to a gold standard (positive in both an antigen test and singleplex PCR test), multiplex real-time PCR test had an 88% sensitivity and 98% specificity across all three parasitic species. It was concluded that multiplex PCR assays provided an alternative method for multiplex antigen assay tests, which has not been developed yet (only singleplex is clinically used) (22).

In another study by Verweij and coworkers (2004), *E. histolytica*, *G. lamblia*, and *C. parvum* were identified in a multiplex real-time PCR assay. This study found a 100% specificity across all three parasitic species when compared to the gold standard of microscopic testing, and that multiplex assays were just as effective as single assays. These authors concluded that multiplex real-time PCR is a feasible technique for the routine diagnosis of these parasitic infections (52).

Duplex real-time PCR (the simultaneous testing of two parasitic species) to identify *E. histolytica*, *Cryptosporidium* spp. and *D. fragilis* was also found to be superior to microscopy in terms of sensitivity. Bruijnesteijn and coworkers (2009) conducted a study in northwest Europe and they concluded that real-time PCR improved diagnostic yield by 18% (10).

A recent study by Tuniuchi and coworkers (2011) found that by using two multiplex real-time PCR assays, it was possible to test for a total of seven parasites. These investigators found between an 83-100% sensitivity and specificity across all seven parasites and concluded that the use of molecular methods is a highly useful as laboratories are quickly losing expertise to detect parasites using stool microscopy (50).

The purpose of this research was to determine if real-time multiplex PCR could be configured to simultaneously amplify and detect the presence of *G. lamblia*, *D. fragilis*, and *S. stercoralis*. The sensitivity, specificity, advantages, limitations and practical uses of this technique were evaluated against the current standard method of diagnosis, light microscopy carried out at the Texas DSHS parasitology laboratory

MATERIALS AND METHODS

Study Specimens

The data for this project were taken from the Texas DSHS Parasitology Database. Fecal samples from around the state were collected and sent to the medical parasitology team at DSHS. They receive a total of about 7,500 fecal specimens a year (C. Snider, personal communication). Thirty-two samples (each no more than two years old) were selected from fecal specimens reserved at DSHS from 2010 to 2012. Each sample contained at least one of three parasites: *G. lamblia*, *D. fragilis* or *S. stercoralis*. These samples were sent to the molecular biology team to be tested by multiplex RT-PCR.

Protocol for Multiplex Real-Time PCR

The detailed protocol used for real-time PCR analysis can be found in Appendices A, B, C, and D . Appendix A details the method of extraction, storage, and setup for multiplex RT-PCR. Appendix B details the specific concentrations of the mastermix used in multiplex RT-PCR. Appendix C contains the specific runtime instructions for the LightCycler PCR instrument. Appendix D specifies the probe/primer sequences.

Validation of Parasite Probe/Primer

The probes and primers for each of the three parasites were first validated in an uniplex assay to ensure that they worked. The first step was vertrel extraction of the parasites from the stool sample by the addition of lysis buffer, and followed by centrifugation of the sample. The second step involved the extraction of parasitic DNA from that sample using the MagNA Pure instrument. Lastly a sample plate, which contained extracted parasitic DNA and a probe for a single parasite, was loaded into the LightCycler 480 PCR machine for analysis. Each uniplex assay was configured such that each parasite was matched to its respective probe/primer mix. A negative control consisted of deionized water and a positive control consisted of an unrelated parasite.

Validation of Parasite Probe/Primer in Multiplex Assays

The procedure for multiplex assays differed in that a mastermix was added during the last step that contained multiple probes for different parasites, instead of just a single probe.

RESULTS

Demographic analyses

The parasitic infection rates for refugees in the study population as determined by microscopy can be found in Figure 4. Forty-one percent were infected with *D. fragilis*, 31% were infected with *G. lamblia* and 13% were infected with *S. stercoralis*. Nine percent were co-infected with *D. fragilis* and *G. lamblia*, and 6% were co-infected with *D. fragilis* and *S. stercoralis*. Figure 5 represents the top 2 countries of origin for the refugees included in the study population. Seventy-six percent of the refugees came from one of two countries, Burma or Cuba. The remaining 25% of refugees came from 6 other countries; Eritrea, Congo, Chad, Nepal, Zimbabwe and one unknown country. The gender distribution of the sample population was 1.4 male to female ratio as shown in Figure 6. Figure 7 shows the distribution of refugees broken down by age group. Forty-seven percent were between the ages of 0 and 15, 31% were between the ages of 21-40 and 22% were agres 56 and above. Figure 8 represents the city of residence for the refugees in the study population. Thirty one percent lived in Houston, 22% lived in Dallas, 16% lived in Lewisville, and 12% lived in Amarillo. The last 19% lived in four other cities; Abilene, Austin, Carrolton and one unknown city.

Uniplex PCR Results

The parasitic infections identified in the 32 refugees that made up the study population by light microscopy included 9 infections with *G. lamblia*-only, 3 infections with *G. lamblia* and *D. fragilis*, 12 infections with *D. fragilis*-only, 4 infections with *S. stercoralis*-only, and 2 infections with *S. stercoralis* and *D. fragilis* (Table 1).

The results for the uniplex assay of *G. lamblia* are shown in Figure 9. The probe for this parasite fluoresces at 610 nm. In Figures 9 to 16, the color green indicates a negative result (no detection of the parasite) and the color red is a positive result (a detection of the parasite). The two flat green lines represent the positive and negative controls. The negative control consisted of water while the positive control contained the DNA of an unrelated parasite (a hookworm). Both controls did not fluoresce. Twelve of 12 samples tested for *G. lamblia* were positive. Based on these results, it was concluded that the uniplex assay to identify *G. lamblia* was successful.

The results for the uniplex assay of *D. fragilis* are represented in Figure 10. The probe for this parasite fluoresces at 440 nm. Seventeen samples were tested for *D. fragilis*, and as shown by the red curves in Figure 10, 14 of 17 samples tested positive. The five green curves represent three samples and two controls that tested negative for *D. fragilis*. Even though 100% of samples were not positive, it was concluded that the uniplex assay to identify *D. fragilis* was successful.

The results for the uniplex assay of *S. stercoralis* are shown in Figure 11. This probe fluoreseces at 640 nm. Six of 6 samples tested for *S. stercoralis* were negative. Based on these results, it was concluded that the uniplex assay to identify *S. stercoralis* was unsuccessful.

The percent agreement between the uniplex molecular assay and microscopy is given in Table 2. In the case of *G. lamblia*, there was a 100% agreement between microscopic and molecular methods. In the case of *D. fragilis*, there was an 82.4% agreement between the two methods. In the case of *S. stercoralis*, it was concluded that the probe/primer mix did not work as intended.

Multiplex PCR

The multiplex assays were configured such that each sample was tested with a probe/primer mix for *G. lamblia* and *D. fragilis*, since the *S. stercoralis* probe or primer was not working as intended. Theoretically, if a sample only had *G. lamblia*, then it would only fluoresce at a wavelength of 610 nm, and if a sample only had *D. fragilis*, it would fluoresce at a wavelength of 440nm. If a sample had both parasites, it would fluoresce at both wavelengths.

The results for the duplex assay of *G. lamblia* at a wavelength of 610 nm as shown in Figure 12. Just like the uniplex assay, 12 of 12 samples for *G. lamblia* tested positive and 2 of 2 controls tested negative. It was important to verify that the 9 *G. lamblia*-only samples did not fluoresce at a wavelength of 440 nm. As shown in

Figure 13, 9 of 9 *G. lamblia*-only samples were negative at a wavelength of 440 nm. The two red curves represent 2 of 3 samples from individuals that were co-infected with both parasites. The third sample, from a refugee that was co-infected with both parasites, was not detected.

The results of the duplex assay to detect *D. fragilis* at a wavelength of 440nm are depicted in Figure 14. Like the uniplex assay, the multiplex assay detected 14 of 17 samples that contained *D. fragilis*, including two samples that were co-infected with *G. lamblia* and *D. fragilis* and two samples that were co-infected with *S. stercoralis* and *D. fragilis*. The five green curves represent the two negative controls plus the three samples that tested negative for *D. fragilis*. It was important to then verify that the 12 *D. fragilis*-only samples and the 2 *D. fragilis* and *S. stercoralis* samples did not fluoresce at a wavelength of 610 nm. Figure 15 shows that the 12 *D. fragilis*-only samples and the 2 *D. fragilis* and *S. stercoralis* samples were negative at a wavelength of 610 nm. The three red curves represent 3 of 3 samples that were co-infected with *D. fragilis* and *G. lamblia*. These results agree with the previously mentioned studies.

A summary of the agreement between multiplex PCR and microscopy is displayed in Table 3. Nine of nine samples that were positive microscopically for *G. lamblia*, fluoresced at the expected wavelength of 610 nm and were not detected at a wavelength of 440 nm. All 3 samples, in which both *G. lamblia* and *D. fragilis* were present by microscopy, were detected at 610 nm and 2 of the samples were also detected at a wavelength of 440 nm. Twelve of 14 samples microscopically

confirmed to be positive for *D. fragilis*-only or *D. fragilis* and *S. stercoralis* fluoresced at 440 nm and did not fluoresce at 610nm. Finally, the same results were obtained when the 3 samples containing *D. fragilis* and *G. lamblia* were tested a second time.

DISCUSSION

To summarize, the demographic analysis of the 32 refugees, the majority had single parasitic infections with *G. lamblia* or *D. fragilis*. Three-quarters of the refugees came from one of two countries – Burma or Cuba . There was a 1.4 to 1 male to female ratio. Forty-seven percent of refugees were children in the 0-15 age group and 31% were young adults between the ages of 21-40. Slightly more than half of the refugees resided in Houston or Dallas.

This work resulted in the development of a successful multiplex assay to identify two important intestinal parasites in Texas refugees – *G. lamblia* and *D. fragilis*. For *G. lamblia*, there was 100% agreement between uniplex and multiplex assay results with microscopy results, and 100% agreement between multiplex assay results with microscopy results for co-infection with *D. fragilis*. The molecular assays developed had a 0% false positive rate for detection of *G. lamblia* or co-infection of *G. lamblia* with *D. fragilis*. For *D. fragilis*, there was 82.4% agreement between uniplex and multiplex assay results with microscopy results, and a 66% agreement between multiplex assay results and microscopy results for co-infection

with *D. fragilis* and *G. lamblia*. These assays also had a 0% false positive rate for detection of *D. fragilis* or co-infections of *D. fragilis* and *G. lamblia*.

The results of this investigation were consistent with the results found by other investigators such as Tuniuchi and coworkers (2011), Bruijnesteijn and coworkers (2009), and Verweij and coworkers (2004) (10, 50, 52). Uniplex and multiplex RT-PCR are extremely accurate at diagnosing parasitic infection with a quick turn-around time. Of the assays that were successfully validated, they had a high sensitivity and specificity when compared to the golden standard of light microscopy.

There were at least three limitations to this research. First, it was not possible to verify the microscopic identification of parasites in the stool specimens. Second, it was not possible to develop a successful assay for *S. stercoralis* due to a malfunctioning probe or primer. Lastly, the sample size was small.

Possible future studies the author could carry out include repeating the study with a larger sample size to further investigate the discrepancies between microscopy and molecular testing techniques. In addition, the author would like to configure other multiplex assays using additional sets of probe/primer to diagnose a wider range of intestinal parasitic infections. Lastly, two intestinal parasites – *E. histolytica* and *E. dispar* – are indistinguishable by microscopy. The author would like to develop a duplex assay and test its value for distinguishing these two parasites.

Based on these research results, the author would recommend that the DSHS Molecular Biology Laboratory carry out continued research in developing multiplex assays for the identification of three or more intestinal parasites.

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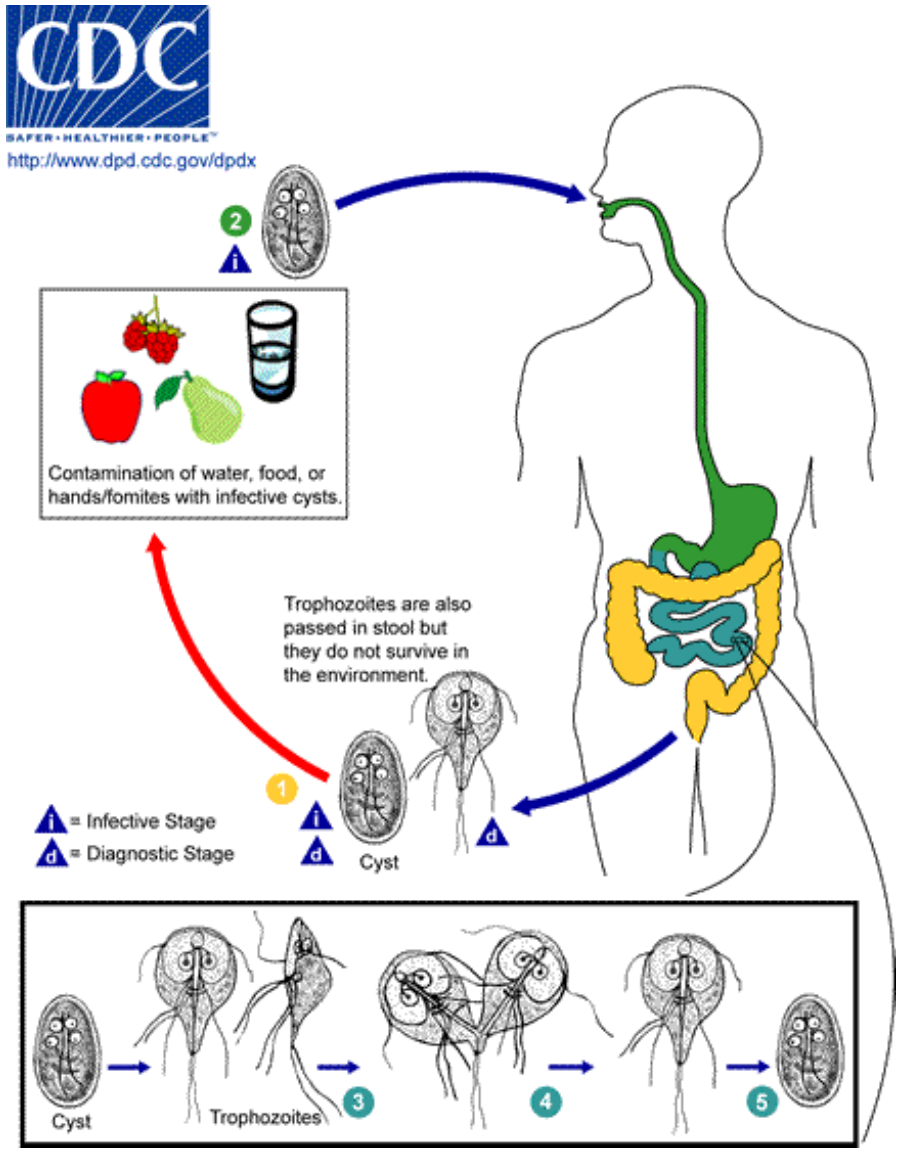
FIGURES AND TABLES

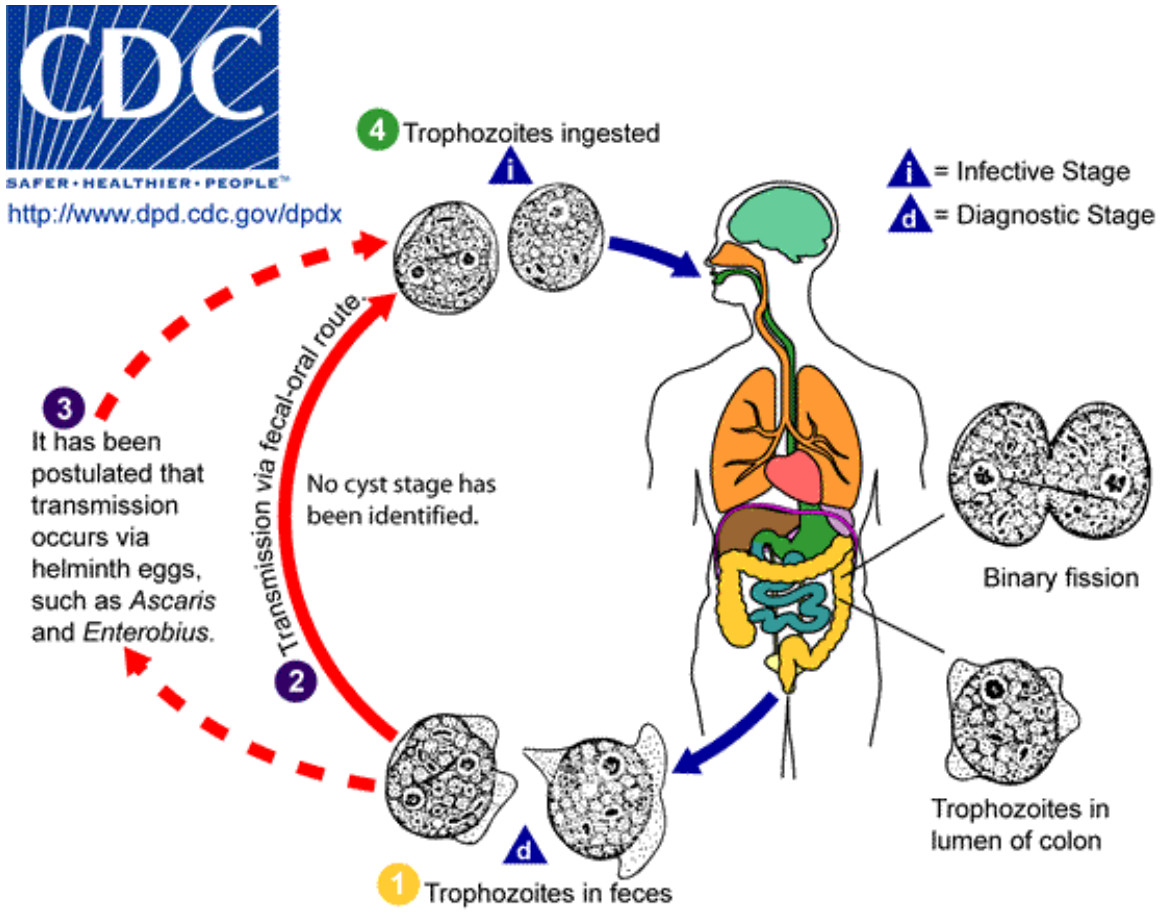
Figure 1. Life cycle of *G. lamblia*



Taken from:

http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/Giardiasis_il.htm

Figure 2. Life cycle of *D. fragilis*



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http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/Dientamoeba_il.htm

Figure 3. Life cycle of *S. stercoralis*

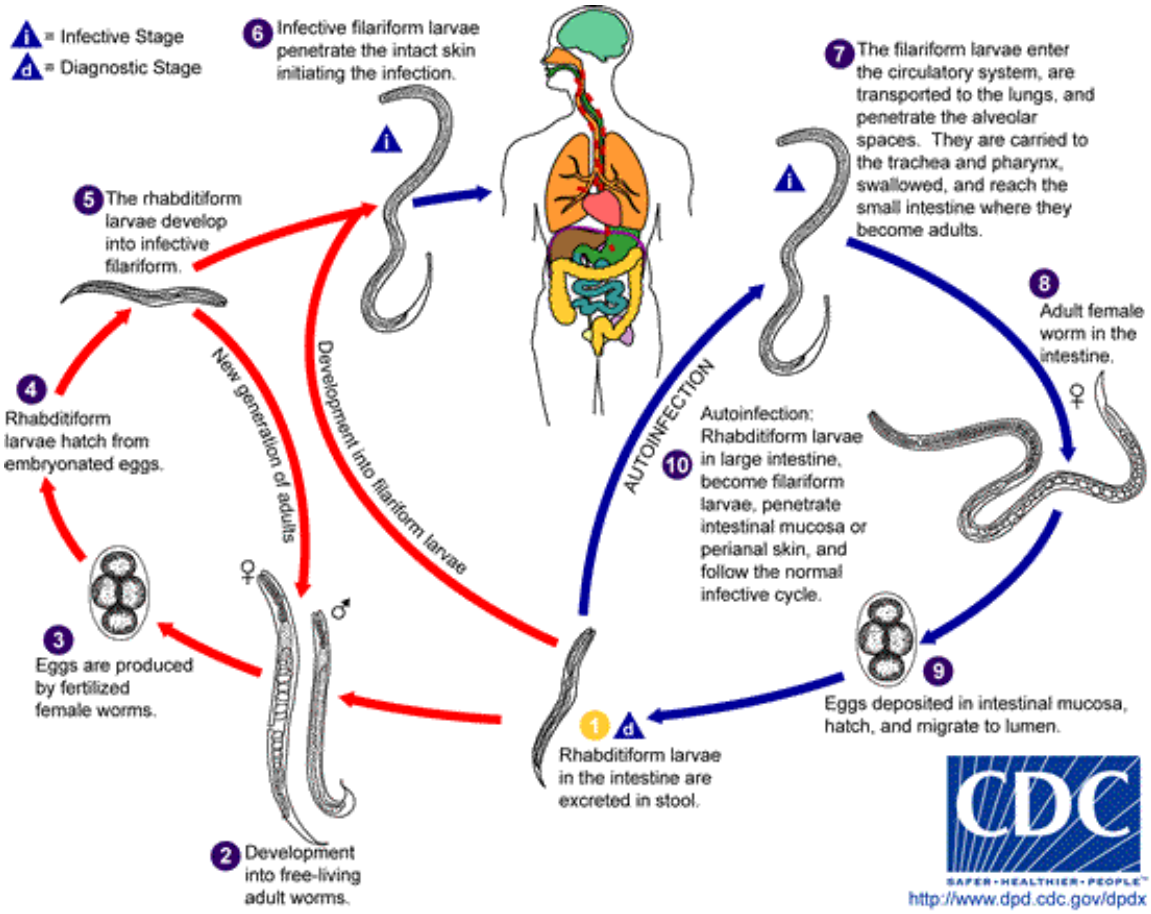

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Figure 4. Parasitic infections in refuges in the study population

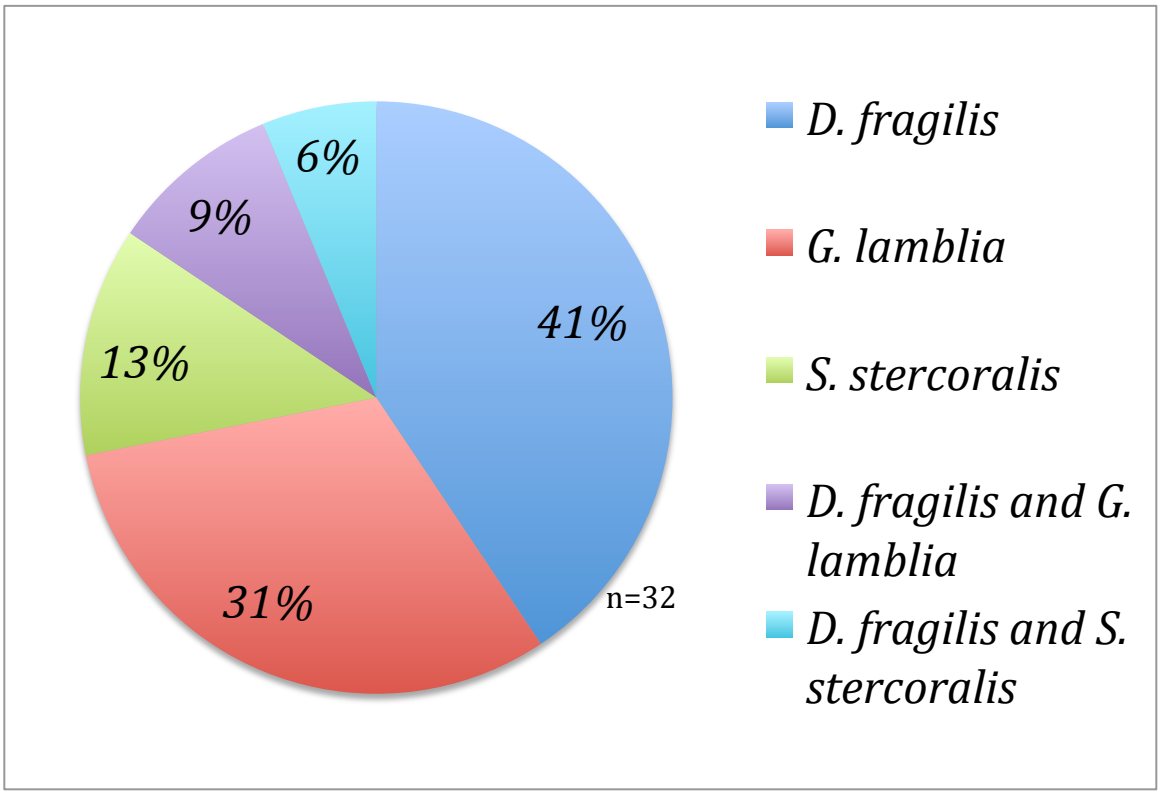


Figure 5. Country of origin of refugees in the study population

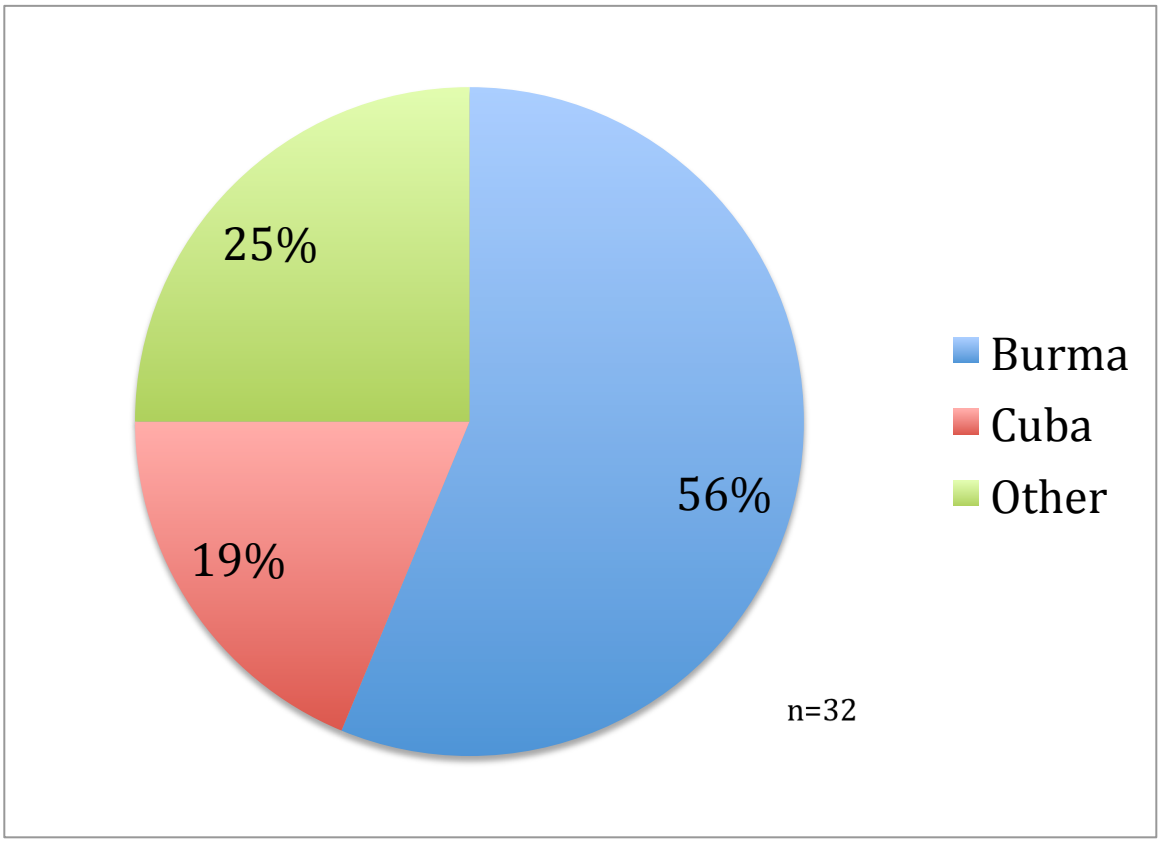


Figure 6. Gender of refugees in the study population

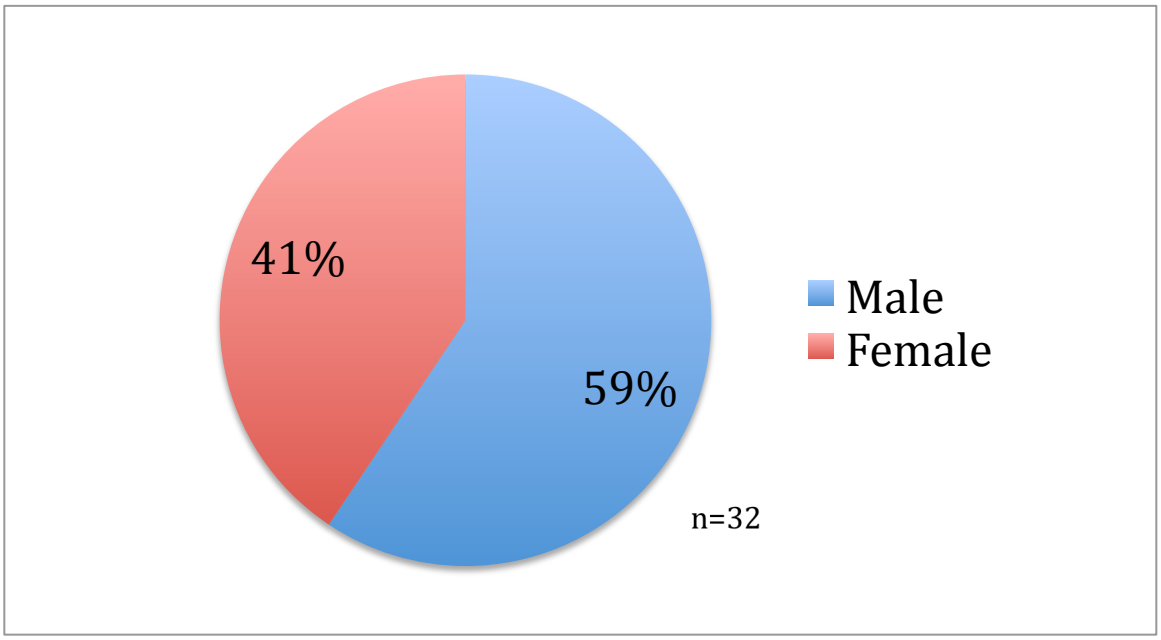


Figure 7. Age of refugees in the study population

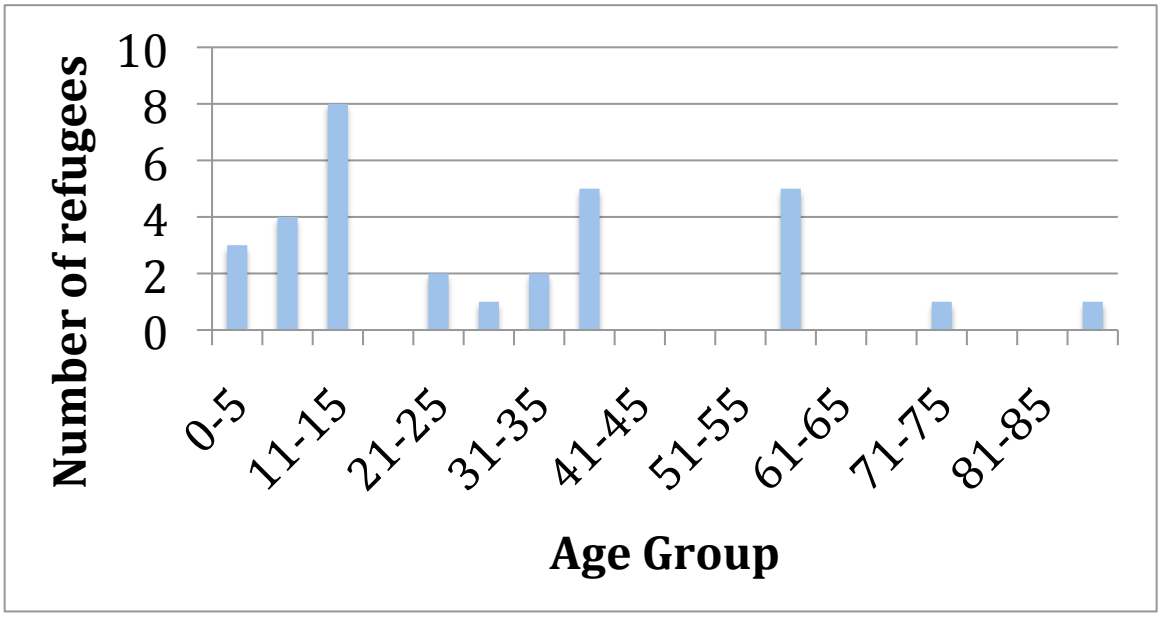


Figure 8. City of residence for refugees in the study population

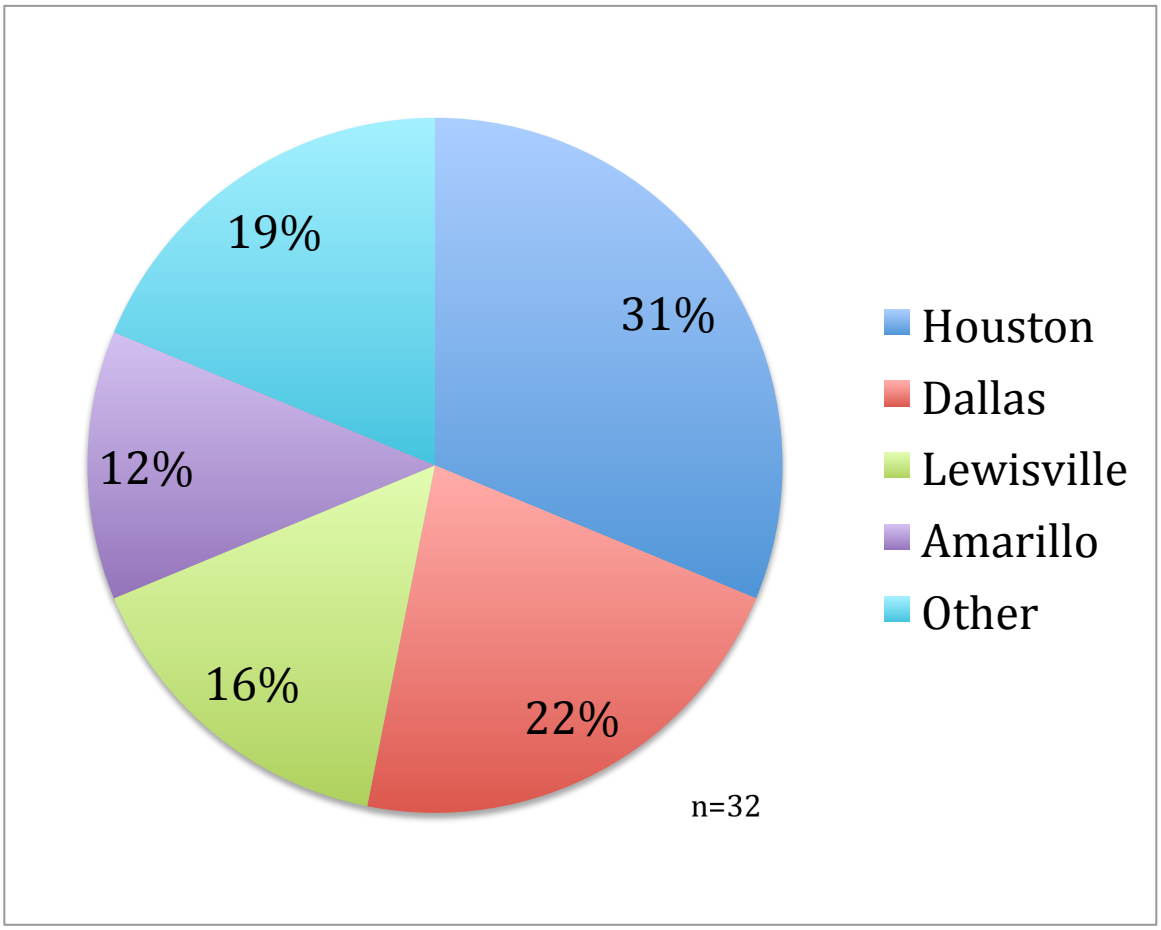


Figure 9. *G. lamblia* uniplex PCR results (610 nm)

Figure 10. *D. fragilis* uniplex PCR results (440 nm)

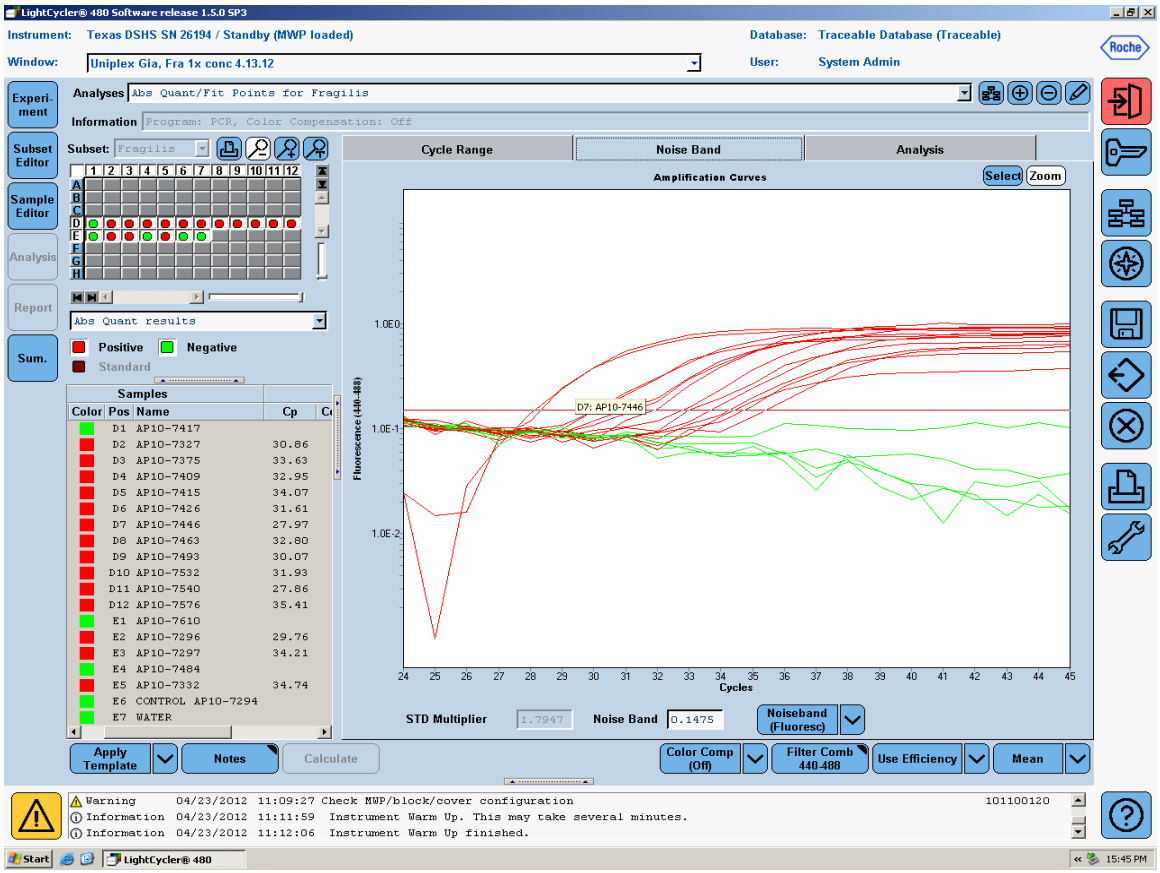


Figure 11. *S. stercoralis* uniplex PCR results (640 nm)

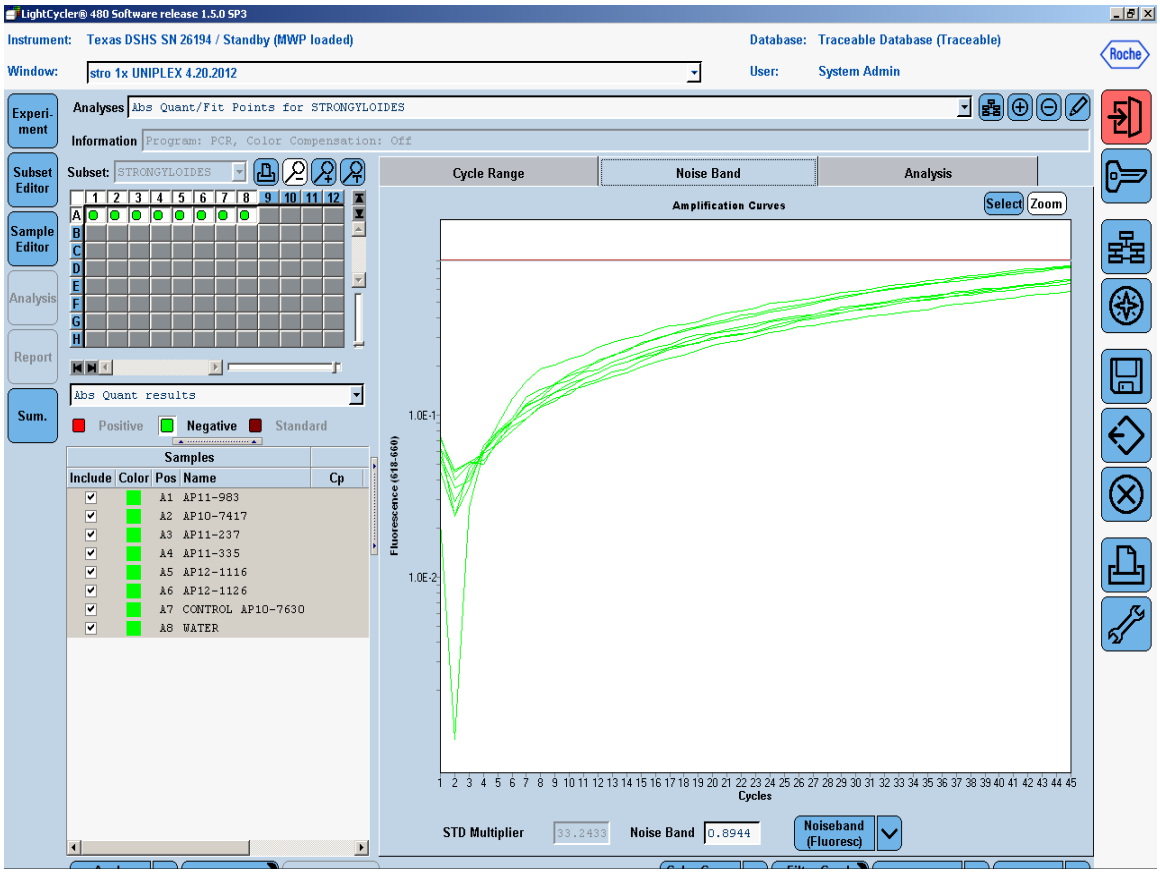


Figure 12. *G. lamblia* multiplex PCR results (610 nm)

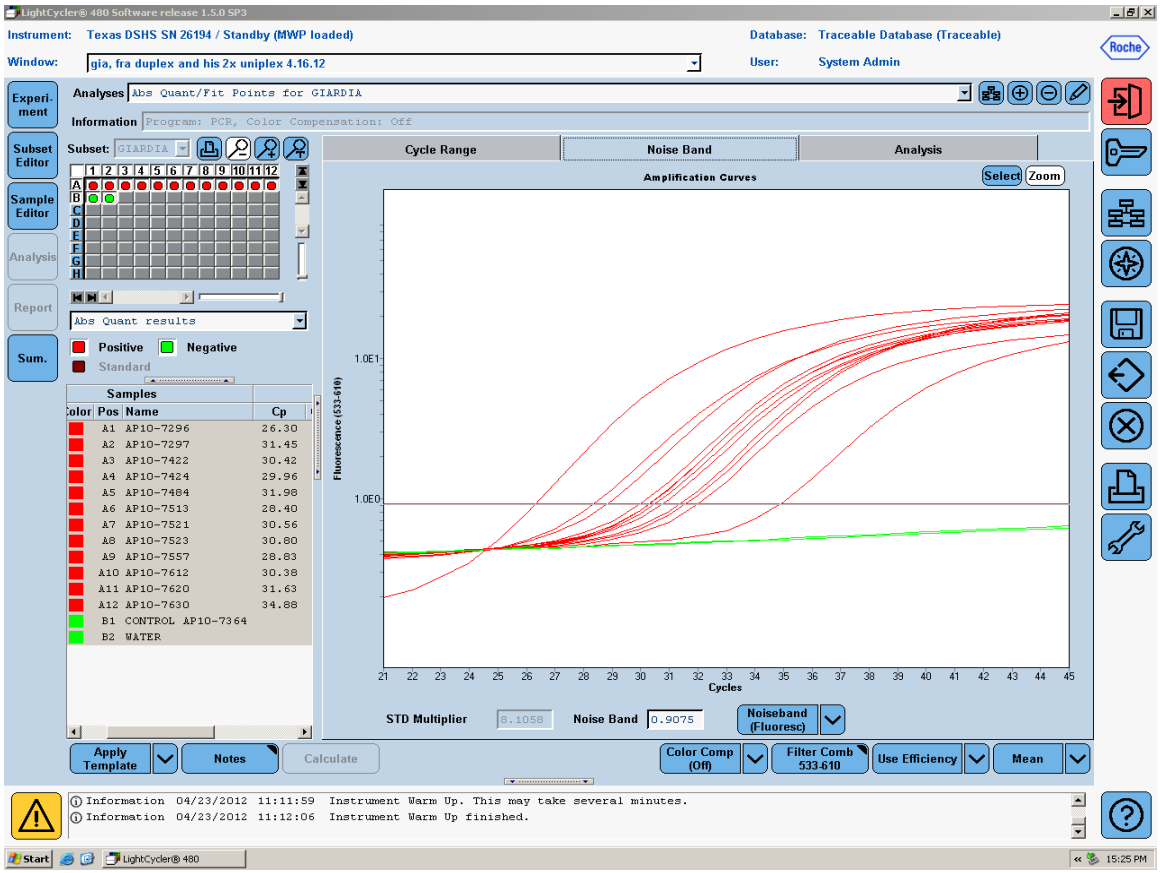


Figure 13. *G. lamblia* multiplex PCR results (440 nm)

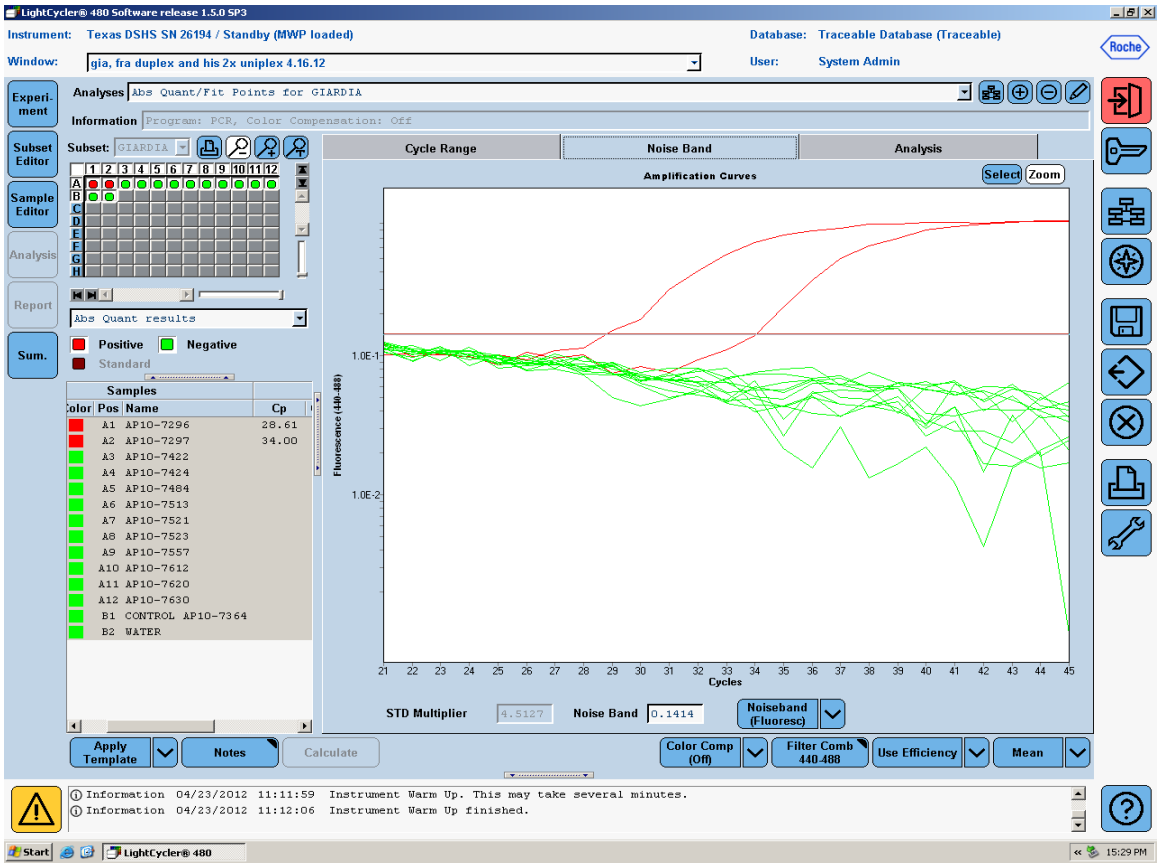


Figure 14. *D. fragilis* multiplex PCR results (440 nm)

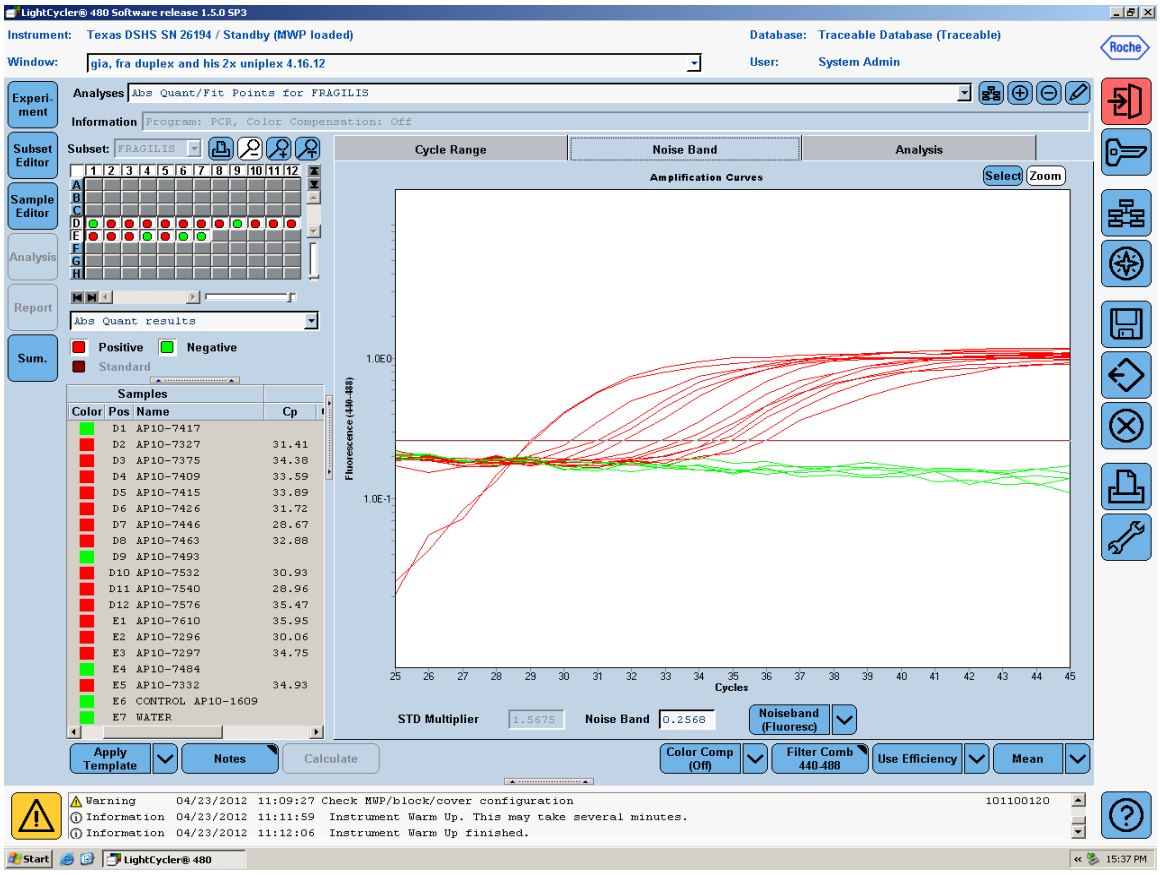


Figure 15. *D. fragilis* multiplex PCR results (610 nm)

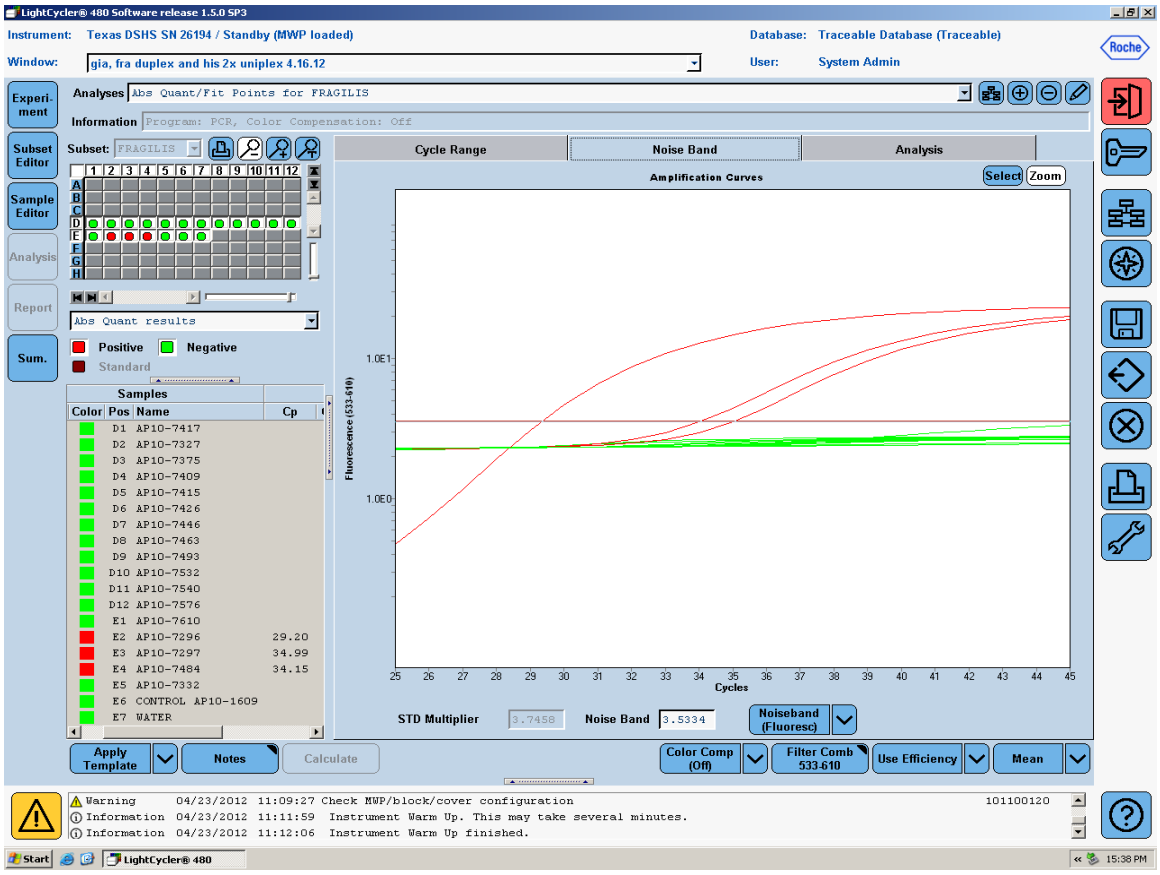


Table 1. Parasitic Infection in study population

Parasite	Count
G. lamblia	**9**
G. lamblia + D. fragilis	**3**
D. fragilis	**12**
S. stercoralis	**4**
S. stercoralis + D. fragilis	**2**

Table 2. Agreement between uniplex PCR and microscopy

Parasite	RT-PCR Positives	Microscopy Positives	Percent Agreement
G. lamblia	12	12	100%
D. fragilis	14	17	82.4%
S. stercoralis	N/A	6	N/A

Table 3. Agreement between multiplex PCR and microscopy

	610 nm (G. lamblia)	440 nm (D. fragilis)
G. lamblia	9/9 (100%)	0/9 (0%)
G. lamblia* + *D. fragilis	3/3 (100%)	2/3 (66.7%)
D. fragilis* or *D. fragilis* + *S. stercoralis	0/14 (0%)	12/14 (85.7%)
D. fragilis* + *G. lamblia	3/3 (100%)	2/3 (66.7%)

APPENDICES

Appendix A. Protocol for Real-Time PCR

A. Vertrel Extraction from Stool Specimen

1. Label microfuge tubes with specimen ID that corresponds to stool specimen.
2. Add 0.5 mL of nuclease-free water into 1.5 mL microfuge tubes.
3. Add 0.5 mL Vertrel into microfuge tubes.
4. If solid stool, add approximately a pea size amount of stool with a disposable transfer loop into appropriate microfuge tube. If liquid stool, add approximately 0.1 mL of stool into appropriate microfuge tube.
5. Vortex each microfuge tube for 1 minute.
6. Centrifuge each microfuge tube for 5 minutes at 2800 RPM at 4˚C.

B. MagNA Pure Extraction from Vertrel Specimen

1. Aliquot 300 µL of lysis buffer into a separate sample microfuge tube.
2. Add 100 µL of aqueous top Vertrel layer from extraction step into sample microfuge tube.
3. Set up MagNA Pure protocols:
    a. Sample volume is 400 µL
    b. Elution volume is 100 µL
4. Load sample and reagents into MagNA Pure and run 'Total_NA_Plasma_External_Lysis" protocol.

C. Real-Time PCR Setup

1. In the PCR setup box:
    a. Create and label tubes for each master mix (Appendix B).

b. Mix by inversion and flicking the tube several times. Centrifuge briefly to collect liquid at bottom of the tube.

2. Dispense 21 µL of each master mix to each appropriate well on a LightCycler 480 (LC480) plate (1 well per specimen per master mix).
3. Add 4 µL of MagNA Pure extractions to appropriate wells.
    a. Be sure to include one positive and negative control for each of the targets.
4. Seal LC480 plate and centrifuge 60 seconds at 3500 rpm.
5. Load plate into LC480.
6. Start the real-time PCR run:
    a. Select 'Run experiment from Template.' Select 'Parasite Panel' experiment file (Appendix C). The settings should be as follows. The machine should have excitation/reading filters at 498/580 nm, X, XXXXXXXXX with the following parameters: melt factor: 1.2; Quant factor: 5; Max integration time: 2 (Parasite Panel Detection Parameters).
    b. Click 'Run'.

D. Reading Results from the LightCycler 480:

1. Click 'Analysis' on left side of page, then 'Fit Points'. Select the sample subset.
2. Select the appropriate wavelength filter. Select the appropriate color compensation file.

3. Adjust left bar on Cycle Range tab to about 5 cycles left from estimated crossing point of first amplification curve.
4. From the Noise Band tab adjust the horizontal bar above the background (<0.2) fluorescence. Click 'Calculate.'

Appendix B. Mastermix Concentrations

Parasite	Amount of forward and reverse primer from 100 µM stock (µL)	Amount of probe from 100 µM stock (µL)	Amount of nuclease free water (µL)
G. lamblia	**6.5**	**2.5**	**91**
D. fragilis	**1.5**	**2.5**	**96**
S. stercoralis	**1.5**	**2.5**	**96**

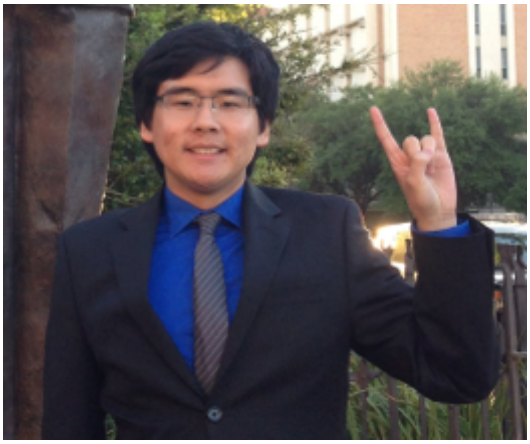
Appendix C: Parasite Panel

1. Program the LighCycler:
    a. Denature (1 cycle)
        i. 95˚C for 10 minutes; 4.4˚C/s ramp rate
    b. PCR (45 cycles)
        i. 95˚C for 10 sec; 4.4˚C/s ramp rate
        ii. 60˚C for 1 minute; 2.2˚C/s ramp rate, single acquisition mode
    c. Cooling (1 cycle)
        i. 40˚C for 10 sec; 1.5˚C/s ramp rate

Appendix D. Probe/primers

1. *G. lamblia*
    a. Giardia-80F
        i. GACGGCTCAGGACAACGGTT
    b. Giardia-127R
        i. TTGCCAGCGGTGTCCG
    c. Giardia-105T
        i. LC610-CCCgCggCggTCCCTgCTAg—BBQ
2. *D. fragilis*
    a. Df-124F
        1. CAACGGATGTCTTGGCTCTTTA
    b. Df0221R
        1. TGCATTCAAAGATCGAACTTATCAC
    c. Df-172revT
        1. 6FAM - CAA TTC TAG CCG CTT AT - MGBNFQ
3. *S. stercoralis*
    a. Stro18S-1530F
        i. GAATTCCAAGTAAACGTAAGTCATTAGC
    b. Stro18S-1630R
        i. TGCCTCTGGATATTGCTCAGTTC
    c. Stro18S-1586T
        i. LC640-ACACACCggCCgTCgCTg—BBQ

AUTHOR BIOGRAPHY



Louis Bian is graduating from The University of Texas at Austin in May 2014 with a B.S. Public Health Honors degree. A student in the Dean's Scholars Honors Program, Louis began research as a freshman in the Freshman Research Initiative. He continued his research the following year with Dr. Cowperthwaite at St. David's NeuroTexas Institute. He completed his public health internship his second year with the Molecular Biology Team at the Texas Department of State Health Services.

Louis was also actively involved in extracurricular programs and organizations on campus. He was a teaching assistant and mentor for three years in the Freshman Research Initiative in Dr. Ruth Shear's Research Methods class and Dr. Greg Palmer's Antibiotics Research Stream. He also underwent a volunteer service trip to Nicaragua to provide disadvantaged children with modern medical care. Additionally, he has volunteered for over 200 hours in various hospitals, over 100 hours in the community, and 60 hours at Camp Reynal (a summer camp for children with serious kidney diseases).

Louis will be attending The University of Texas Southwestern Medical School in Dallas, Texas next year as an M.D. candidate. He hopes to provide the best care possible with a public health perspective in mind.